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Technical Note

The chick embryo chorioallantoic membrane as a model for *in vivo* research on angiogenesis

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ABSTRACT The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane that is commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors. Quantitative or semiquantitative methods may be used to evaluate the amount of angiogenesis and anti-angiogenesis. Thanks to the CAM system, angiogenesis could be investigated in association with normal, inflammatory and tumor tissues, and soluble factors inducing angiogenic or anti-angiogenic effects could be identified. Rabbit cornea provides an alternative *in vivo* system, but CAM appears to be easier to handle and less expensive. Moreover, CAM can be used with very few limitations.

KEY WORDS: *angiogenesis, anti-angiogenesis, chorioallantoic membrane, chick embryo*

Introduction

In vivo and *in vitro* techniques are available for research on the functions of endothelial cells during angiogenesis (Auerbach *et al.*, 1991). Of the *in vivo* techniques, those utilizing chick extraembryonic membranes, namely the chorioallantoic membrane (CAM) and the *area vasculosa*, both of which are copiously vascularized, as well as the one employing the rabbit cornea, i.e. an avascular organ, are the most reliable ones.

Histogenesis and structure of CAM

The allantois is an extraembryonic membrane, derived from the mesoderm, in which primitive blood vessels begin to take shape on day 3 of incubation. On day 4, the allantois merges with the chorion epithelium, derived from the ectoderm, to form the chorioallantois (Romanoff, 1960) (Fig. 1A,B). Primitive vessels continue to proliferate and to differentiate into an arteriovenous system until day 8 (Fig. 2A), thus originating a network of capillaries that migrate to occupy an area beneath the chorion and mediate gas exchanges with the outer environment (Fig. 2B). Rapid capillary proliferation goes until day 11; thereafter, their mitotic index declines just as rapidly, and the vascular system attains its final arrangement on day 18, just before hatching (Ausprunk *et al.*, 1974).

In ovo utilization

Fertilized eggs staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951) are placed into an incubator as soon as embryogenesis starts and are kept under constant humidity at 37°C. At HH stage 20, a square window is opened into the shell after removing 2-3 ml of albumen so that the developing chorioallantois is detached from the shell itself and the underlying CAM vessels are disclosed (Fig. 3A,B). The opening is closed with a cellophane tape and incubation goes on until the day of the experiment.

In vitro utilization

By other methods, the CAM vascular system is displayed in greater detail, except that the embryo and the extraembryonic membranes must be transferred to a Petri dish in the early stages of development, i.e. on days 3 or 4 of incubation (Auerbach *et al.*, 1974) (Fig. 3C). There CAM develops at the top as a flat membrane, reaching the edge of the dish to provide a two-dimensional monolayer onto which grafts can be placed. Because the entire membrane can be seen, rather than just a small portion through the shell window, multiple grafts can be placed on each CAM and photographs can be taken periodically to document vascular changes over time.

Subsequently, several modifications of this method have been described (Dugan *et al.*, 1981; Dunn *et al.*, 1981; Jakobson *et al.*, 1989). Dugan *et al.* (1981) used an inert plastic container (instead

Abbreviations used in this paper: CAM, chorioallantoic membrane; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; EGF, epidermal growth factor; PDGF, platelet derived growth factor.

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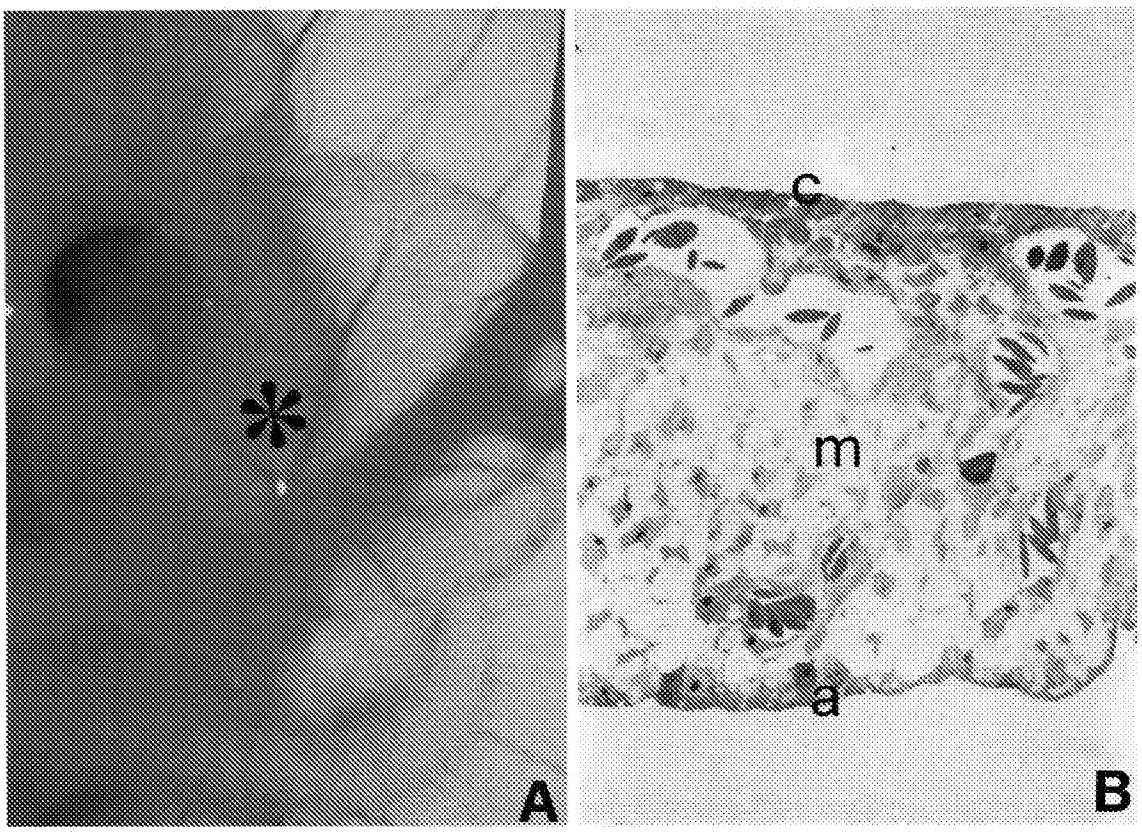

Fig. 1. Macroscopic and and microscopic CAM structure. **(A)** *Allantoic sac (asterisk) of a 5-day embryo showing in ovo distribution pattern of allantoic vessels.* **(B)** *Histological picture of the CAM of 8-day chick embryo showing chorion epithelium (c), intermediate mesenchyme (m) and allantoic endoderm (a). A, x25; B, x160*

of a Petri dish) equipped with a "parafilm" ring (4-5 cm inside depth) to provide support for the embryo and its membranes. Advantages include somewhat longer viability and lower costs, though these are offset by the difficulty of monitoring angiogenesis during incubation and by the fact that one cannot obtain two-dimensional photographs suitable for image analysis.

Testing substances and tissues

The test substance is soaked in inert synthetic polymers laid upon the CAM: Elvax 40 (ethylene-vinyl acetate copolymer) and hydron (a poly-2-hydroxyethyl-methacrylate polymer, HydroMed Sciences) are commonly used. The two polymers were first described and validated by Langer and Folkman (1976): both proved to be biologically inert when implanted onto the CAM and both were found to polymerize in the presence of the test substance, allowing its sustained release during the assay. However, hydron requires the test substance to be added to a solution of hydron and ethanol. When the test pellets are vacuum-dried, ethanol is removed leaving a solid pellet that contains the test substance. If the test material is not compatible with ethanol, Elvax can be used instead. Elvax is dissolved in methylene chloride before the test material is suspended/dissolved in the polymer, after which methylene chloride is removed by vacuum-drying. A more sustained release can be achieved by "sandwiching" the test substance between two Elvax layers. The polymers cause the substance to be released at constant rates (nanograms to micrograms) around-the-clock.

Alternatively, when testing a fluid substance, the latter is inoculated (20-50 µl) directly into the cavity of the allantoic vesicle, so that its activity will develop evenly over the whole vascular area (Ribatti *et al.*, 1987).

Another method has been recently proposed by Nguyen *et al.* (1994): by this method, a collagen gel is conjugated with the testing substance and placed between two pieces of mesh (bottom layer 4x4 mm, upper layer 2x2 mm). The resulting "sandwich" is then placed upon the CAM on day 8 of incubation.

Tissues to be tested are converted under sterile conditions in minute bits (1-2 mm^3) that are gently placed onto the CAM with a fine forceps on day 8-9 of incubation.

When polymers are used in combination with an angiogenic substance, a vasoproliferative response will be recognizable 72-96 h after implantation: the response takes the form of increased vessel density around the implant, with the vessels radially converging towards the centre like spokes in a wheel (Fig. 4A-D). Conversely, when polymers combined with an anti-angiogenic substance are tested, then vessels become less dense around the implant after about 72-96 h, and eventually disappear (Fig. 4E).

Lastly, when the substance is inoculated into the cavity of the allantoic vesicle, then the angiogenic or anti-angiogenic response affects the CAM vessels as a whole.

Evaluating the vasoproliferative response by semiquantitative methods

Several semiquantitative methods are used to evaluate the extent of the vasoproliferative response. One method considers changes in the distribution and density of CAM vessels next to the implant which are evaluated *in vivo* by means of a

stereomicroscope at regular intervals following the graft procedure. The score is 0 when no changes can be seen; it is +1 when few neovessels converge towards the implant, and +2 when a considerable change in the number and distribution of the converging neovessels is observed (Knighton *et al.*, 1977).

By another method, the vasoproliferative response is graded as a vascular index derived from photographic reconstructions. All converging neovessels contained inside a 1 mm-in-diameter ring superimposed upon the CAM are counted: the ring is drawn around the implant in such a way that it will form an angle of less than 45° with respect to a straight line drawn from the implant's centre. Vessels branching dichotomically outside the ring are counted as 2, while those branching inside the ring area counted as 1 (Dusseau *et al.*, 1986).

A third method measures the degree of vasoproliferative response, as evaluated under the stereomicroscope, by an arbitrary 0-to-5 scale. Zero describes a condition of the vascular network that shows no change from the time of grafting; +1 marks a slight increase in the vessel density associated to occasional changes in the course of vessels converging toward the implant; +2, +3, +4 and +5 indicate a progressive increase in vessel density associated with more pronounced changes in their course, while a +5 score also highlights strong hyperemia. A coefficient describing the degree of angiogenesis can also be derived from the ratio of the calculated value to the highest attainable value. Therefore, the coefficient's lowest value is 0 and the highest is 1 (Folkman and Cotran, 1976).

Strick *et al.* (1991) calculate the length of the vessels and express it in terms of index density, i.e. the vessel density relative to a fractional image area of the vasculature. By using the above mentioned method of Nguyen *et al.* (1994), they express the vasoproliferative response after 72-216 h as a percentage of the squares in the upper mesh occupied by neovessels. The effect of the inhibitory substances (placed on the bottom mesh) is quantified by calculating the inhibition of the vasoproliferative response induced by an angiogenic factor such as, for example, the basic fibroblast growth factor (bFGF).

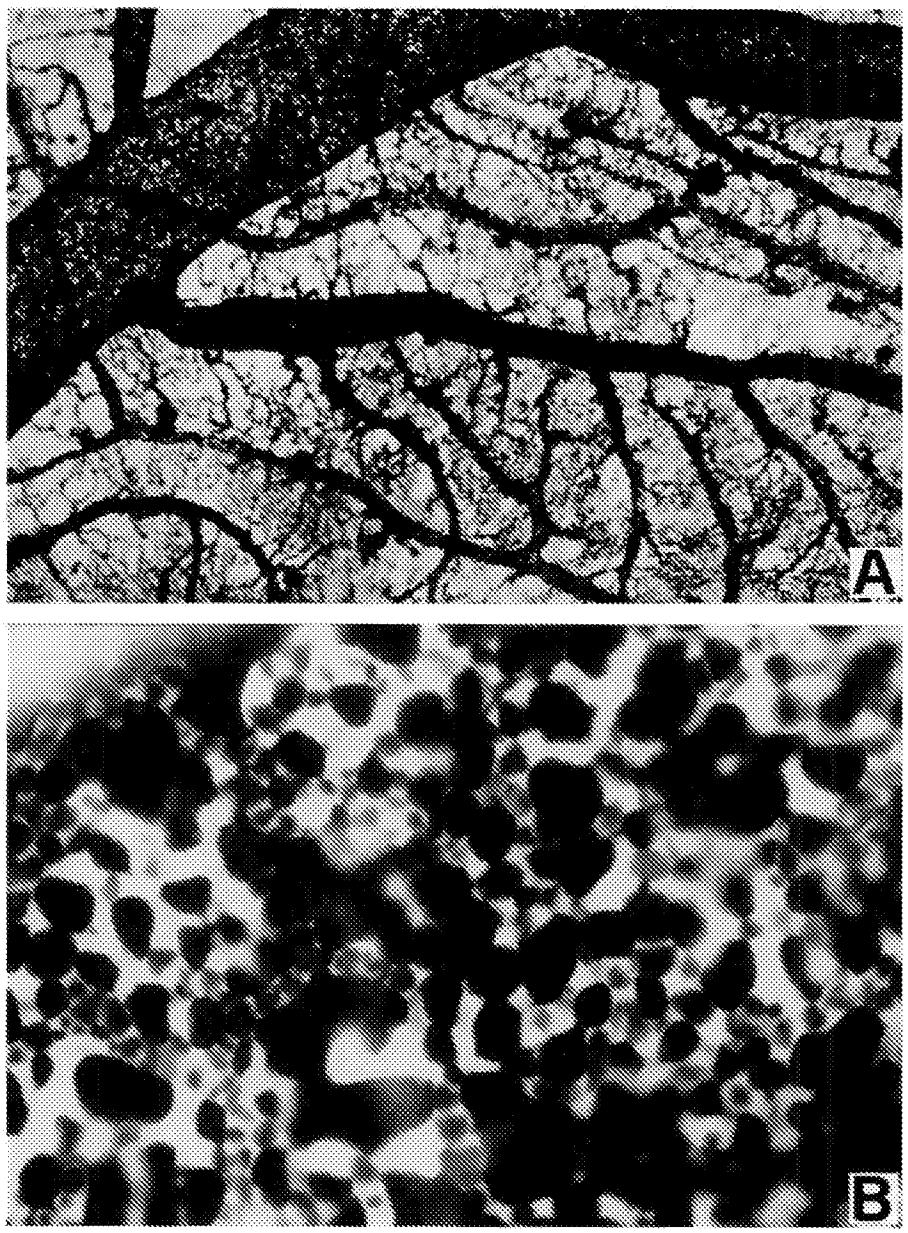

Fig. 2. Macroscopic features of CAM vasculature. (**A**) *Image of the CAM arteriovenous system after an in vivo intravascular injection of India ink.* (**B**) *Image of the CAM superficial capillary network after an in vivo intravascular injection of fluorescein-isothiocyanate-labelled dextran. A, x100; B, x400.*

Evaluating the vasoproliferative response by quantitative methods

Quantitative evaluation of vessel density can be obtained by applying morphometric and planimetric methods to histologic observations of CAM specimens fixed at regular intervals after implantation (Fig. 5). The number of vessels is evaluated as the total number of vessels present in 6 randomly chosen microscopic fields (x200). Vessel density is evaluated by a planimetric method (Elias and Hyde, 1983) which utilizes a square reticule (with 144 points of intersection of 12 lines per side) placed in the eyepiece of a photomicroscope. Six randomly chosen fields per section are observed and the total number of intersection points occupied by transversally sectioned vessels is counted. Vessel density equals this total number, expressed as the percent value of all intersection points. Evaluation of the number and density of vessels should be made by two independent observers and processed statistically.

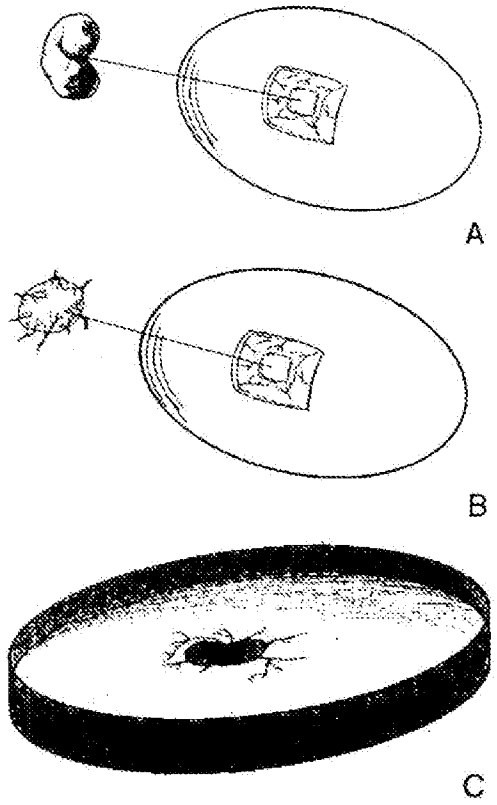

Fig. 3. Different CAM uses. (A,B) *Drawings showing the in ovo use of CAM vasculature for embryonic* **(A)** *or tumoral* **(B)** *tissue grafts.* **(C)** *Drawing showing the in vitro use of CAM vasculature utilizing a Petri dish.*

Another method measures the ^{3}H-thymidine incorporated into the CAM. The amount incorporated into the DNA is expressed as a percentage of total ^{3}H-thymidine present in the CAM homogenate, regardless of the CAM weight and full recovery of applied radioactivity (Thompson *et al.*, 1982; Splawinski *et al.* 1988). Automatic image analysis has also been applied (Jakob and Voss, 1984; Tanara *et al.*, 1986).

The limitations of CAM

CAM finds its main limitation in non-specific inflammatory reactions which may develop from the implant and in turn induce a secondary vasoproliferative response, eventually making it difficult to quantify the primary response that is being investigated (Jakob *et al.*, 1978; Spanel-Borowski *et al.*, 1988). In this respect, a histologic study of CAM sections would help to detect the possible presence of a perivascular inflammatory infiltrate together with a hyperplastic reaction, if any, of the chorion epithelium. In an extensive series of experiments by Jakob *et al.* (1978), a variety of carrier vehicles alone (Millipore filters, fiber glass discs, gelatine and viscose sponges, discs of filter paper, agarose and polyacrylamide gels), as well as natural egg components (egg shell membrane, coagulated albumin and coagulated yolk) produced a number of inflammatory reactions.

However, the possibilities of causing a non-specific inflammatory reaction are much lower when the implant is made very early in CAM development, since at that time the host's immune system is relatively immature. Earlier lymphoid cells deriving from the yolk sac and spleen are usually recognizable in the thymus on day 8 and in the Fabricius bursa on day 11 (Leene *et al.*, 1973).

There are three more drawbacks to the CAM assay. First, the test material is placed on pre-existing vessels, so neovascularization and the re-arrangement of vessels can hardly be distinguished from each other (Knighton *et al.*, 1991). Second, timing of the CAM angiogenic response is essential. Many studies determine angiogenesis after 24 h, a time at which there is no angiogenesis, but only vasodilatation. It would be worthwhile to point out that measurements of vessel density are really measurements of visible vessel density, and that the distinction between vasodilatation and neovascularization is not easy to make. To circumvent this drawback it is useful to utilize sequential photography to document new vessel formation. Third, often polymers do not adhere to the CAM surface. To circumvent this drawback, Folkman (1984) hydrated the testing material with 5 μl H_2O on sterile coverslide glass, which is turned over and placed onto the CAM on day 9-10 of incubation. The possible angiogenic response is evaluated 48 h later.

A similar approach has been tried by Wilting *et al.* (1991, 1992); they used culture coverslide glasses (Thermanox) 4-5 mm-in-diameter, on which 5 μl of several angiogenic factors were placed. Glasses were turned over and placed onto the CAM on day 9 of incubation, and the angiogenic response was evaluated 96 h later.

Saline solutions should be avoided, in that the hyperosmotic effect of crystal salts may damage the chorion epithelium and induce fibroblast proliferation (Wilting *et al.*, 1991). This implies that the substance be used at concentrations of picograms to milligrams; higher concentrations would indeed cause the hyperosmotic effect (Wilting *et al.*, 1992).

Extracts from a number of normal cells (Barnhill *et al.*, 1984; Leibovich *et al.*, 1987; Auerbach *et al.*, 1991; Ribatti *et al.*, 1991) and tissues (Ausprunk *et al.*, 1977) have been found to stimulate vessel growth when implanted onto CAM.

Ausprunk *et al.* (1977) compared the behavior of tumor grafts (see below) to grafts of normal adult and embryo tissues. In tumor tissue, pre-existing blood vessels in the tumor graft disintegrated within 24 h after implantation, and revascularization occurred by penetration of proliferating host vessels into the tumor tissue. By contrast, pre-existing vessels did not disintegrate in the embryo graft and anastomosed to the host vessels with almost no neovascularization. In adult tissues, pre-existing graft vessels disintegrated (although this process was slower than in tumor vessels) and did not stimulate capillary proliferation in the host. Lastly, tumor vessels did not reattach to those of the host.

These studies suggest that only tumor grafts are capable of stimulating formation of new blood vessels in the host, thus gaining their blood supply.

Angiogenesis induced by chronic inflammatory tissues

Chronic inflammation is accompanied by neovascularization as an obligatory means to sustain it. For example, in arthritis new

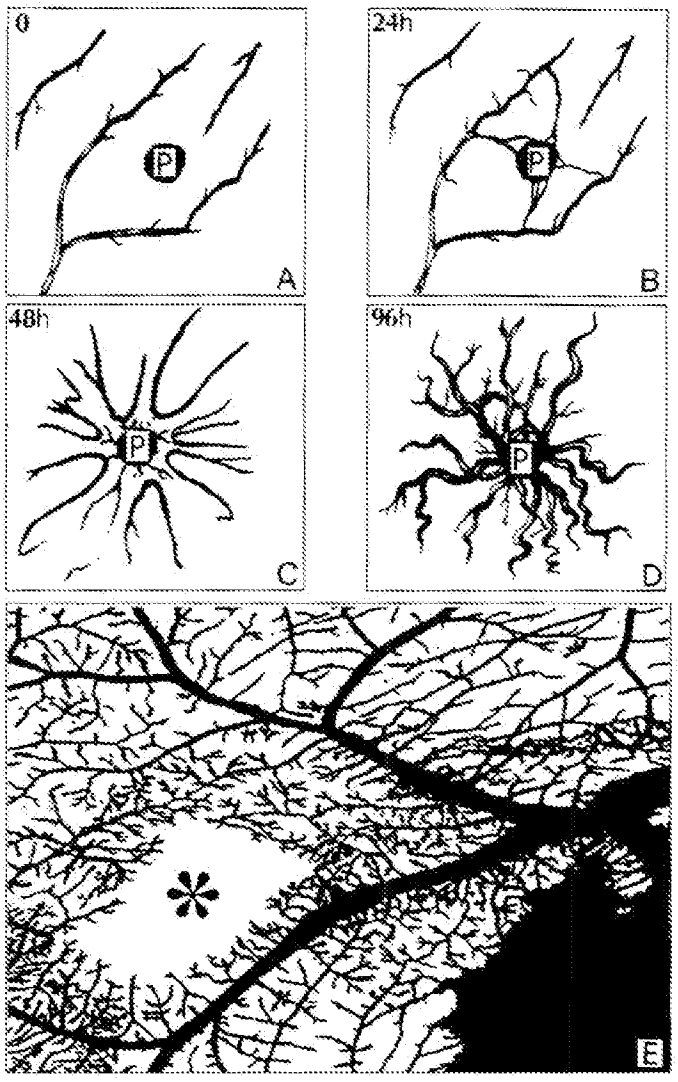

Fig. 4. The angiogenic and the angiostatic responses of CAM vasculature (A-D) *Drawings of the time-course of CAM response to a polymer (P) soaked with an angiogenic substance. (Modified from Folkman and Cotran, 1976).* **(E)** *Drawing of an avascular zone (asterisk) in a CAM exposed to an anti-angiogenic substance.*

capillary blood vessels invade the joint and destroy cartilage; in diabetes, new capillaries in the retina invade the vitreous and cause blindness.

Hypoxia has been identified as a key regulator of angiogenesis and a potential source of angiogenic factors, such as the vascular endothelial growth factor (VEGF).

The angiogenic activity of several chronic inflammatory processes, such as rheumatoid arthritis (Brown *et al.*, 1980), diabetic retinopathy (Hill *et al.*, 1983) and of hypoxia (Dusseau and Hutchins, 1988) has been tested onto CAM.

Tumor angiogenesis

Tumor angiogenesis is a prerequisite for tumor growth, while the growth of new blood vessels appears to be "switched on" at some point after a tumor becomes established (Folkman, 1995; Vacca *et al.*, 1995). Metastasis, too, is angiogenesis-dependent: correlations between microvessel density in tumor and future metastases have been reported by various investigators (Gasparini, 1994; Vacca *et al.*, 1994).

Knighton *et al.* (1977) first described the onset (from day 8 to 16) of tumor vascularization in the CAM having used implants of fresh Walker 256 carcinoma. Chick capillaries proliferated in the vicinity of the tumor graft about 24 h after implantation, but capillary sprouts did not penetrate the graft until approximately 72 h later. During the avascular interval, tumor diameter did not exceed 1 mm, but grew rapidly during the first 24 h following capillary penetration.

The angiogenic activity of other malignant neoplasms, such as melanoma (Auerbach *et al.*, 1976; Klagsbrun *et al.*, 1976), meningioma (Klagsbrun *et al.*, 1976; Splawinski *et al.*, 1988), glioblastoma (Klagsbrun *et al.*, 1976), lymphomas (Mostafa *et al.*, 1980; Ribatti *et al.*, 1990), endometrial carcinoma (Splawinski

TABLE 1

STUDIES DEMONSTRATING THE ANGIOGENIC ACTIVITY OF VARIOUS SUBSTANCES ONTO CAM

Authors	Substance
Fraser *et al.* 1979	Adenosindiphosphate (ADP)
Mc Auslan *et al.*, 1983	Copper
Form and Auerbach, 1983	Prostaglandin E_2 (PGE_2)
Thompson *et al.*, 1982, 1985	Fibrin degradation products
Mc Auslan *et al.*, 1983	Heparin*
Ribatti *et al.*, 1987	Heparin*
West *et al.*, 1985	Degradation products of hyaluronic acid
Morris *et al.*, 1988	Phorbol esters

*When absorbed on methylcellulose discs and applied onto the CAM, heparin exerts an anti-angiogenic effect (Jakobsson and Hahnenberger, 1991).

TABLE 2

STUDIES DEMONSTRATING THE ANGIOGENIC ACTIVITY OF SEVERAL GROWTH FACTORS ONTO CAM

Authors	Growth factor
Esch *et al.*, 1985 Wilting *et al.*, 1991 Olivo *et al.*, 1992 Ribatti *et al.*, 1995	Basic fibroblast growth factor (bFGF)
Fett *et al.*, 1985 Wilting *et al.*, 1991 Olivo *et al.*, 1992	Angiogenin
Leibovich *et al.*, 1987 Olivo *et al.*, 1992	Tumor necrosis factor alpha (TNF-α)
Roberts and Sporn, 1989 Stewart *et al.*, 1989 Wilting *et al.*, 1992 Wilting *et al.*, 1992, 1993	Transforming growth factor beta (TGF-β) Epidermal growth factor (EGF) Platelet derived growth factor (PDGF) Vascular endothelial growth factor (VEGF)

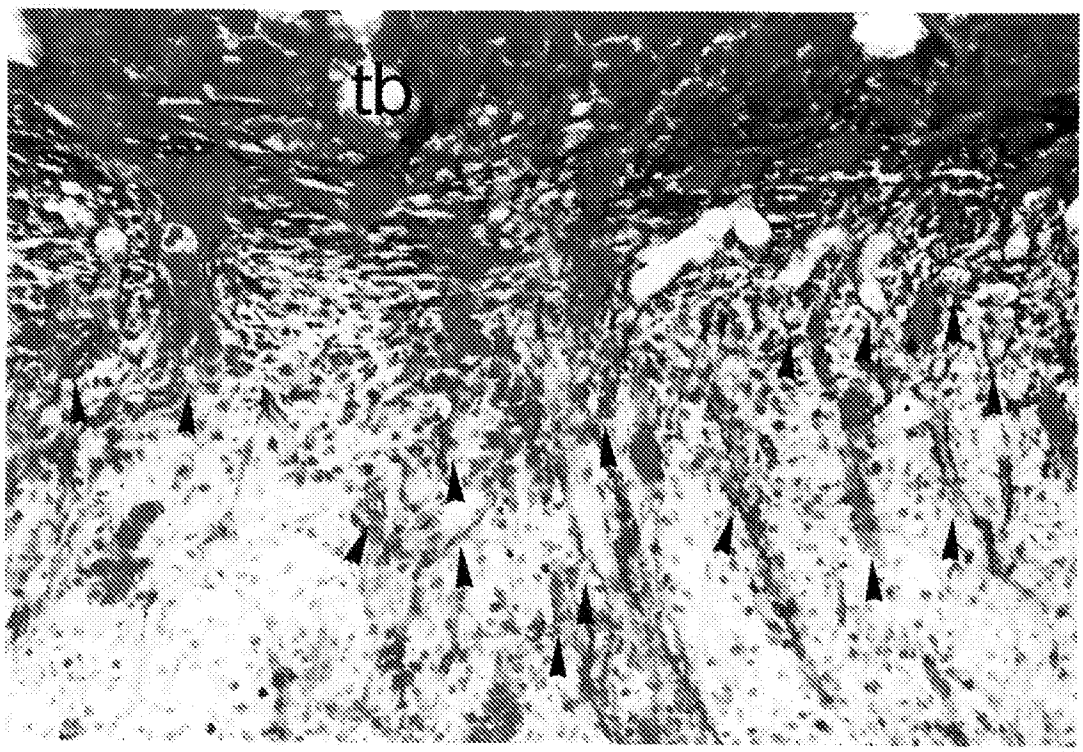

Fig. 5. The vasoproliferative response of CAM vasculature to a tumor implant. *Histological picture showing the positive angiogenic response in the CAM's intermediate mesenchyme on incubation day 12, 96 h after implantation of a lymph node biopsy involved by a high grade B-cell non-Hodgkin's lymphoma. New blood vessels (arrowheads) converge toward the tumor base (tb).*

et al., 1988; Ribatti *et al.*, 1996a) and head squamous cell carcinoma (Petruzzelli *et al.*, 1993), has been tested onto the CAM.

Testing angiogenic substances

Several substances cooperate in making up the natural microenvironment in which angiogenesis takes place. For example, "the microenvironment of a solid tumor *in vivo* can be defined as the space between the basement membrane of the microvessels and the surface of the neoplastic cells. It is characterized by two components – the stroma and the interstitial fluid" (Gullino, 1995).

Studies demonstrating the angiogenic activity of various substances upon the CAM are summarized in Table 1. Generally, all the substances in the list are regular constituents of both the stroma and the interstitial fluid.

TABLE 3

STUDIES DEMONSTRATING THE ANTI-ANGIOGENIC ACTIVITY OF VARIOUS SUBSTANCES ONTO CAM

Authors	Substance
Eisenstein *et al.*, 1975	Chondrocyte derived inhibitor
Taylor and Folkman, 1982	Protamine
Folkman *et al.*, 1983	Heparin or heparin fragments + cortisone
Crum *et al.*, 1985	Heparin+11-hydrocortisone or 17-hydroxyprogesterone
Ingber and Folkman, 1988	Analogs of proline
Maragoudakis *et al.*, 1988	Inhibitors of basement membrane biosynthesis
Folkman *et al.*, 1989	Beta cyclodextrin tetradecasulfate (TDS) + angiostatic steroids
Oikawa *et al.*, 1989	Retinoids
Maione *et al.*, 1990	Platelet factor 4
Oikawa *et al.*, 1990	Vitamin D3 analogues
Ingber *et al.*, 1990	Angiostatin
Woltering *et al.*, 1991	Somatostatin
Wilks *et al.*, 1991	Suramin+angiostatic steroids
O'Reilly *et al.*, 1994	Angiostatin
Sasisekharan *et al.*, 1994	Heparinase
Ribatti *et al.*, 1996b	Interferon alpha 2a

Testing angiogenic factors

Reports on the angiogenic activity of several growth factors on CAM are summed up in Table 2.

Among such factors, VEGF specifically induces blood vessel growth (Wilting *et al.*, 1992, 1993). Concerning the pivotal angiogenic role of another growth factor, the basic fibroblast growth factor (bFGF), Wilting *et al.* (1993) stated that: "...bFGF strongly increases the proliferation rate evenly throughout the stroma of the CAM, which means that is mainly fibrocytes and only to a minor degree endothelial cells that respond to the factor". Recently we have clearly demonstrated that endogenous bFGF has a rate-limiting role in the vascularization of the CAM (Ribatti *et al.*, 1995).

Testing anti-angiogenic substances

Angiogenesis appears to depend on the balance of several stimulating and inhibiting factors. Inhibition of blood vessel growth, i.e. anti-angiogenesis, or else stimulation of angiogenesis-inhibiting factors, would seem to provide a strategy for preventing both the growth of tumors and other angiogenesis-dependent diseases (Auerbach and Auerbach, 1994).

Studies demonstrating the anti-angiogenic activity of several substances on the CAM are summarized in Table 3.

Concluding remarks

CAM is widely utilized as an *in vivo* system to study angiogenesis and anti-angiogenesis. Although rabbit cornea is used just as often as an *in vivo* system, CAM offers the advantage of being simpler to use and less expensive. Moreover, unlike the rabbit cornea, the CAM system allows several assays to be carried out simultaneously in the same embryo, especially when the *in vitro* approach is used instead. On the other hand, there are only very few restrictions to using CAM, essentially due to: a) non-specific inflammatory reactions that may develop with an attending secondary stimulation of angiogenesis and, b) pre-existing vessels may be present which make it hard to distinguish the extent of angiogenesis and anti-angiogenesis.

An important step forward in using the CAM system, which we ourselves are working on, consists in isolating the endothelial cells from the CAM vessels and culturing them to test angiogenic or anti-angiogenic factors. A parallel research on these factors upon the CAM will enable us to understand to what extent the periendothelial microenvironment provided by CAM as a whole is capable of affecting angiogenesis or anti-angiogenesis.

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